JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 531–540
0021-9193/83/080531-10$02.00/0

Vol. 155, No. 2

Amplifiable Resistance to Tetracycline, Chloramphenicol, and Other Antibiotics in *Escherichia coli*: Involvement of a Non-Plasmid-Determined Efflux of Tetracycline

ANTHONY M. GEORGE[1] AND STUART B. LEVY[1,2]*

Departments of Molecular Biology and Microbiology[1] and of Medicine,[2] Tufts University School of Medicine, Boston, Massachusetts 02111

Received 4 January 1983/Accepted 10 May 1983

Increasing levels of resistance to tetracycline and to a number of other unrelated antibiotics, including chloramphenicol, β-lactams, puromycin, and nalidixic acid, occurred in *Escherichia coli* after 50 to 200 generations of growth in the presence of subinhibitory concentrations of tetracycline or chloramphenicol. In the absence of selective pressure, resistances fell to low levels within 100 generations of growth. This amplification of resistance was observed in laboratory and naturally occurring *E. coli* strains as well as in *polA* and *recA* strains. With the exception of previously identified *cmlA* and *cmlB* mutations, tetracycline or chloramphenicol resistances were not P1 transducible. Coincident with the emergence of resistance was the appearance of a previously cryptic energy-dependent efflux system for tetracycline. The expression of resistance phenotypes and the tetracycline efflux system were temperature sensitive at 42°C.

Chromosomal genes for low-level resistance (at doses of 1 to 5 μg/ml) to tetracycline, chloramphenicol, and penicillins have been described in *Escherichia coli* K-12 (33). More than one resistance phenotype may be elaborated by a single gene; many unlinked loci have been identified, and these are P1 transducible to sensitive strains by direct and indirect selection (33, 34). These chromosomal mutations include *cmlA* (resistance to chloramphenicol [34]), *cmlB* (resistance to chloramphenicol and tetracycline [33]), *lon* (resistance to chloramphenicol and tetracycline [33]), and several other loci that have not been precisely mapped or identified, but promote low-level resistance to one or several antibiotics (33, 38).

Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids. However, high-level chloramphenicol-resistant mutants were isolated by Cavalli and Maccacaro (8, 9) by growth of first-step mutants (resistant to chloramphenicol at 5 μg/ml) in successively higher increments of the drug and ultimately approaching the solubility limit of chloramphenicol at about 2 mg/ml. Frequent back-mutation to low-level resistance occurred in the absence of drug selection. Cross-resistance to other antibiotics was not reported.

In this paper we describe the amplifiable, high-level coordinate expression of resistance to tetracycline and chloramphenicol with attendant resistance to several other structurally unrelated drugs. Specific assays and the structural diversity of these antibiotics suggest that detoxification is an improbable mechanism of resistance. For at least tetracycline, we report the detection of an energy-dependent efflux system that appears with amplified resistance. This finding invites speculation about the origins and relatedness of chromosome- and plasmid-mediated resistance mechanisms.

MATERIALS AND METHODS

Bacterial strains. All strains were *E. coli* K-12, except ML308-225 (39). A complete list of strains and constructions is given in Table 1.

Bacteriophage. P1 *vir* was obtained from A. Wright. λ and f2 phage were used for sensitivity testing as previously described (29).

Media. MacConkey agar (Difco Laboratories, Detroit, Mich.) contained 1% lactose and antibiotics at the concentrations specified for individual experiments. L broth and L agar were prepared as defined previously (21). Minimal A medium or agar (27) contained 0.5% sugars and was supplemented with amino acids (20 μg/ml) and antibiotics, where required, at the following concentrations: 500 μg of streptomycin sulfate and 60 μg of nalidixic acid or rifampicin per ml. Other antibiotics were used at concentrations specified below. All antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.), except minocycline (gift of Lederle Laboratories, Pearl River, N.Y.).

Selection of Tet and Cml mutants. Single-step Tet or Cml mutants were selected on MacConkey agar plates that contained antibiotic at 5 μg/ml. Often a wide variation in numbers of resistant colonies was obtained for different strains and for the same strains in different experiments. The spontaneous mutation rate

TABLE 1. *E. coli* K-12 strains

Strain[a]	Characteristics	Source or reference
DO-23	*uncB402 argE3 thi-1 rpsL xyl mtl galK supE44* λ	F. Gibson via J. Davies (formerly AN382)
AG100	Same as DO-23, but *uncB*$^+$ and Δ(*gal-uvrB*) λ$^-$	This paper; spontaneous *uncB*$^+$ revertant; (P1 · [Δ(*gal-uvrB*)] JF222 → DO-23)
AG102	Same as AG100, but expresses Tc10^{rb}	This paper
AG106	Same as AG100, but expresses Tc50^r	This paper
AG122	Same as AG100, but expresses Cm10^r	This paper
AG123	Same as AG100, but expresses Cm20^r	This paper
AG126	Same as AG100, but expresses Cm50^r	This paper
D1-188	Same as DO-23, but contains R222	This laboratory
PA309	*gal-6 rpsL9 argH1 his-1* λ$^-$ *gyrA tonA2 thr-1 mtl ara-13 xyl-7 malA1 lacY1 thi-1 trp leu-6*	4
GMS407	*argE3 lacY1 galK2 manA4 mtl-1 tsx-29 supE44 uidA*	30 via CGSC[c]
DW1021	*rpsE gyrA100* Δ*lac-74 thi-1*	19
DW1030	Same as DW1021, but *recA13*	19

[a] This table does not contain many referenced and constructed strains that were used in single experiments. These appear in the appropriate sections of the text.

[b] Phenotypes given in the table only represent the levels of Tc^r or Cm^r, based on selection with the indicated concentration (micrograms per milliliter) of tetracycline or chloramphenicol. Each of these mutants expresses other antibiotic phenotypes at various levels (for complete profiles, see Table 3).

[c] CGSC, *E. coli* Genetic Stock Center (B. Bachmann, curator).

was measured in the absence of drug selection by the fluctuation test of Luria and Delbruck (22).

Amplification of resistance. Amplification of resistance was achieved by two methods.

(i) **Method A.** Cultures were grown in L broth at 37°C to the late exponential or early stationary phase. About 10^9 cells were spread over the surface of MacConkey agar plates containing tetracycline or chloramphenicol at 5 μg/ml. These plates were incubated at 37°C for 24 to 60 h. Loopfuls of resistant cells from colonies on these plates were transferred to plates that contained tetracycline or chloramphenicol at 10 μg/ml. These were incubated at 37°C again for 24 to 60 h. This stepwise process was repeated in increments of 10 μg/ml (for tetracycline) or 20 μg/ml (for chloramphenicol). Eventually, cells resistant to at least 100 μg/ml were obtained.

(ii) **Method B.** The isogenic pair DW1021 and DW1030 (*recA*) were spread on plates containing tetracycline or chloramphenicol (see above). Suspensions of these first-step resistant cells were diluted in L broth containing tetracycline or chloramphenicol at 5 μg/ml and grown for many generations with frequent subculture to fresh antibiotic medium. After various generation times, samples were removed, diluted, and spread on MacConkey agar plates containing tetracycline or chloramphenicol at 5 μg/ml. These plates were incubated at 37°C for several hours and then replica-plated to plates that contained successively higher concentrations of either antibiotic. Resistance was scored as the highest antibiotic concentration that permitted >80% survival of the colonies from the master plate (which contained between 100 and 500 colonies) after 24 h at 37°C. After 200 generations, cultures were diluted and transferred to L broth without antibiotic and grown for 100 generations with frequent subculture to fresh medium. Again, samples were removed at various times, diluted, plated on antibiotic-free media, and then replica-plated and scored on antibiotic-containing plates.

MIC. (i) Method A. Minimum inhibitory concentrations (MICs) were determined in L broth after 18 h at 37°C with aeration, beginning with an inoculum size of 10^4 CFU/ml (26).

(ii) Method B. The gradient plate method (6) was used to estimate MICs when it was necessary to screen many strains and antibiotics. Plates were inoculated from sterile, cotton-tipped swabs dipped in suspensions of cells that were adjusted to an absorbancy at 530 nm of 0.2. MICs were determined from the limit of confluent growth after 40 h at 30 or 37°C.

P1 transduction. The use of P1 *vir* for generalized transduction has been described previously (11, 21).

Enzyme assays. A rapid colorimetric assay (3) was used to detect chloramphenicol acetyltransferase activity and was verified by the inclusion of enzyme-containing and enzyme-free strains in the assay. β-Lactamase activity was determined by the following qualitative plate test (31). Molten agar (1.5%) in water was cooled to 45°C, phenol red and penicillin G were added to final concentrations of 0.01 and 0.3%, respectively, and the pH was adjusted to 7. Plates were poured and set at room temperature and used within several hours. Colonies from overnight L agar plates were patched onto the indicator plates. D15-12 [ML308-225(RP1), Tc^r Km^r Ap^r] and D20-6 [χ984(pBR322), Tc^r Ap^r] were included as positive controls. The appearance of yellow zones around the colony patches within 60 min was considered positive for β-lactamase activity.

Antibiotic transport assays. Cells were grown in L broth at 35, 37, or 42°C with aeration to an absorbancy at 530 nm of 0.8 (four doublings) and then harvested (3,000 × *g*, 5 min) and washed twice in prewarmed 50

mM potassium phosphate buffer (pH 6) (pH 7.2 for [^{14}C]penicillin G uptake experiments) containing 1 mM $MgSO_4$ and suspended in the same buffer to an absorbancy at 530 nm of 4.0. These cell suspensions were kept at 35, 37, or 42°C for not more than 30 min before being used in uptake assays or were partially starved by incubating suspensions in assay medium for 1 to 2 h. Accumulation of [7-^{3}H]tetracycline (0.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.), [7-dimethyl-^{14}C]minocycline (12.2 mCi/mmol; gift of Lederle Laboratories), [1-^{14}C]penicillin G (100 mCi/mmol; Penicillin Assays Inc.), and (dichloroacetyl-[1,2-^{14}C]chloramphenicol (gift from New England Nuclear Corp.) by whole cells was determined at 35, 37, or 42°C by our membrane filtration method as described previously (24, 27). At concentrations in excess of 10 μM, radiolabeled antibiotics were supplemented with unlabeled antibiotic. Exogenous 20 mM lactate or glucose was used as the energy substrate, and 2 mM 2,4-dinitrophenol (DNP; General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio) or 25 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (Sigma) was used as the energy inhibitor.

The preparation of everted membrane vesicles was essentially as described previously (25), except that cells were grown in minimal A medium supplemented with 0.5% glucose and 5% L broth. Vesicles were suspended in 10 mM Tris buffer (pH 7.2) containing 140 mM KCl, 0.5 mM dithiothreitol, and 10% (vol/vol) glycerol and stored frozen at −70°C until needed. Transport assays with vesicles (0.7 mg of membrane protein per ml) were performed at 30°C in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM $MgSO_4$.

Definitions. In the context of this study, "amplification" defines incremental elevation of a drug resistance phenotype. Low-level tetracycline or chloramphenicol resistance (Tc^r or Cm^r, respectively) is defined as resistance to 1 to 10 μg/ml; intermediate resistance is defined as resistance to 10 to 50 μg/ml; and high-level resistance is defined as resistance to >50 μg/ml. Numerals indicate the level of resistance achieved by drug selection; for example, a strain exhibiting Tc10^r is resistant to tetracycline at 10 μg/ml in complex medium. Resistant mutants isolated by tetracycline or chloramphenicol selection are given the phenotypic characters Tet or Cml in accordance with the recommendations of this journal. However, in view of the cross-resistances expressed by these mutants (see Table 3), the designations do not infer resistance to only the selective agent.

RESULTS

Characterization of resistance phenotypes. Spontaneous resistance to tetracycline at 5 μg/ml was found initially in the *uncB* mutant DO-23 at a frequency of 10^{-9} to 10^{-8} (26). No single-step mutations to Tc10^r were ever observed, even after 96 h at 37°C, from inocula of 10^{10} cells. Second-step mutation to Tc10^r was achieved in subsequent passages at a frequency of 10^{-7} to 10^{-6}. The ability to isolate these more highly resistant derivatives was not related to the *uncB* mutation, since the resistance remained intact in an *uncB*$^+$ revertant. Moreover, we obtained first-step (tetracycline at 5 μg/ml) and then second-step (tetracycline at 10 μg/ml) Tet mutants from DO-23 *uncB* or *uncB*$^+$ and from the related strains DO-21 (formerly AN180[7]) and DO-22 *uncA* (formerly AN120[7]). We then tested a number of unrelated *E. coli* K-12 and wild-type strains and obtained similar results, indicating that, whereas the *uncB* mutation may have facilitated the isolation of Tet mutants (26), such mutants were obtainable from all *E. coli* strains examined. Many independent Tc^r clones were tested for cross-resistance to chloramphenicol. All first-step Tet mutants were also resistant to chloramphenicol at 10 μg/ml.

Recognition of collateral Tc^r and Cm^r in these mutants prompted us to examine the effect of chloramphenicol selection. By repeating the selection process with agar plates supplemented with chloramphenicol (5 μg/ml), we observed an approximately 10-fold higher spontaneous mutation rate to Cm^r than we had seen for Tc5^r by tetracycline selection, and single-step Cm10^r was also detected. Cross-resistance in Cml and Tet first-step mutants was not wholly reciprocal. Whereas all Tet mutants expressed Cm10^r, only 10 to 60% of Cml mutants expressed Tc5^r.

To quantify the spontaneous mutation rate we performed a Luria-Delbruck fluctuation test, which is based upon the premise that mutation to resistance could occur independently, and with large variability, in any generation before contact with the selective agent (22). Variation was observed in 10 independent cultures of three strains so tested, and the spontaneous mutation rate was computed for each strain (Table 2). There were similar mutation rates to Cm5^r and Tc2.5^r, but 10- to 100-fold lower mutation rates to Tc5^r.

Amplification and stability of resistance phenotypes. Single-step Tet and Cml mutants resistant to tetracycline or chloramphenicol at 5 μg/ml were transferred sequentially to plates containing higher concentrations of either antibiotic. Strains could be so adapted to Tc100^r or Cm100^r and higher. Stepwise amplification with chloramphenicol could be achieved in larger increments (20 μg/ml) than for tetracycline (10 μg/ml) and in less time (24 to 48 h at 37°C for chloramphenicol, 48 to 60 h at 37°C for tetracycline). All *E. coli* strains tested could be made resistant in this way, including *E. coli* C600 (2), the non-K-12 strain ML308-225, a wild-type *E. coli* (SLH1699), and a *polA* K-12 strain that produced high-level resistant mutants.

The isogenic strain pair DW1021 and DW1030 (*recA*) was chosen to examine the effect of the *recA* gene on (i) amplifiable resistance under conditions of steady-state antibiotic concentration (5 μg/ml) over many generations of growth

TABLE 2. Spontaneous mutation rates for some *E. coli* K-12 strains[a]

Culture conditions[b]	Spontaneous mutation rate		
	DO-23	DW1021	DW1030
Chloramphenicol (5)	$5.3 \times 10^{-8} \pm 2.7 \times 10^{-8}$	$1.3 \times 10^{-8} \pm 0.9 \times 10^{-8}$	$1.0 \times 10^{-8} \pm 0.9 \times 10^{-8}$
Tetracycline (2.5)	$3.1 \times 10^{-7} \pm 0.7 \times 10^{-7}$	$3.0 \times 10^{-8} \pm 3.5 \times 10^{-8}$	$1.7 \times 10^{-8} \pm 1.2 \times 10^{-8}$
Tetracycline (5)	$5.7 \times 10^{-9} \pm 1.5 \times 10^{-9}$	$2.1 \times 10^{-10} \pm 2.1 \times 10^{-10}$	$2.8 \times 10^{-10} \pm 3.4 \times 10^{-10}$

[a] Spontaneous mutation rate represents mutations per bacterium per generation. For each strain, 10 independent cultures were grown to the stationary phase in 10 ml of L broth at 37°C, beginning with 200 cells per ml. Samples were spread on antibiotic-containing plates and diluted for viable counting on antibiotic-free plates. The number of resistant CFUs from the 10 cultures was averaged and normalized to the viable counts. The spontaneous mutation rate was derived from the equation $r = aN_t\ln(N_tCa)$, where a is the number of mutations per bacterium per generation, r is the average of the number of resistant bacteria in a limited number of samples, N_t is the total number of viable bacteria at time t, and C is the number of independent cultures (22).

[b] Culture was in MacConkey agar containing a drug at the indicated concentration (micrograms per milliliter).

in liquid medium, and (ii) the stability of resistance upon subculture of resistant cells into drug-free medium. The first conclusion from this experiment was the quantitative similarity of amplification and loss of amplification in the *recA*$^+$ and *recA* pair (Fig. 1). (When high-level Tet and Cml mutants of DW1030 were checked to ensure that they retained the *recA* mutation through the amplification process, they were indeed still *recA* as determined by UV sensitivity). As noted earlier, growth in the presence of either antibiotic produced concomitant resistance to the other drug, and, as will be described below, to other antibiotics as well. Two hundred generations of growth in L broth supplemented with either tetracycline or chloramphenicol at 5 μg/ml elicited resistance to about 140 or 120 μg of chloramphenicol per ml and to 60 μg of tetracycline (Fig. 1). Since only 200 generations had elapsed, the forward rate of amplification must have occurred at a much higher frequency than would be expected from a series of spontaneous mutations.

Upon growth in nonselective conditions, cells lost high-level resistance to both antibiotics after 100 generations (Fig. 1), but low-level Tc^r and Cm^r (5 to 10 μg/ml) remained even after 300 generations.

Multiplicity of resistance phenotypes. In view of the cross-resistance to tetracycline and chloramphenicol, we examined the susceptibility of Tet and Cml mutants of DW1021 and DW1030 to eight other antibiotics. Stepwise amplification with either tetracycline or chloramphenicol elicited the following resistance phenotypes: rifampicin (Rf^r), nalidixic acid (Na^r), puromycin (Pu^r), ampicillin (Ap^r), cephalothin (Kf^r), penicillin G (Pc^r), and minocycline (Mc^r) (Table 3). Resistance to the aminoglycoside antibiotics streptomycin and kanamycin was not detected.

Pc^r was strikingly higher than Ap^r and Kf^r in all chloramphenicol- or tetracycline-amplified mutants; but Pc^r and Ap^r increased in the same ratio in Tet and Cml mutants, although the levels reached were lower in the *recA* derivatives. The trend in Kf^r was clearly different; in contrast to

TABLE 3. MICs for derivatives of DW1021 and DW1030

Strain	Culture conditions[a]	MICs (μM)[b]								
		Chloramphenicol	Tetracycline	Minocycline	Penicillin G	Ampicillin	Cephalothin	Puromycin	Rifampicin	Nalidixic acid
DW1021		10	0	0	0	0	0	90	0	1,420
AG342	Tetracycline (10)	180	50	40	320	40	60	550	20	>2,160
AG345	Tetracycline (50)	230	140	70	480	50	80	>1,100	20	>2,160
AG352	Chloramphenicol (10)	140	10	30	300	30	50	390	10	1,530
AG355	Chloramphenicol (50)	190	30	30	430	40	60	950	20	>2,160
DW1030		10	0	0	0	0	0	60	0	1,170
AG362	Tetracycline (10)	160	50	40	170	10	40	520	10	1,290
AG365	Tetracycline (50)	260	130	60	310	30	70	640	10	>2,160
AG372	Chloramphenicol (10)	140	10	30	180	10	30	490	10	1,380
AG375	Chloramphenicol (50)	250	30	40	280	20	40	540	10	1,560

[a] See footnote *b* of Table 2 (tetracycline at 10 μg/ml = 23 μM; chloramphenicol at 10 μg/ml = 31 μM).

[b] MICs were determined by the limits of confluent growth on antibiotic gradient plates (see the text). The results were originally tabulated in micrograms per milliliter and then converted to micromolar to the nearest multiple of 10. All strains listed in the table were completely sensitive to streptomycin and kanamycin.

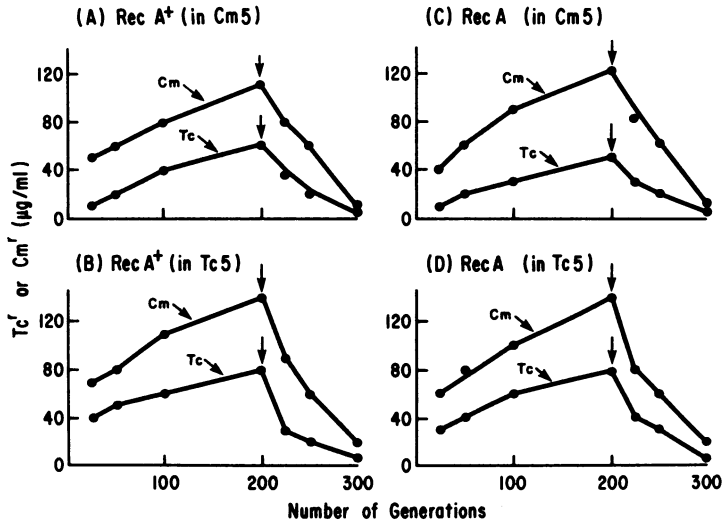

FIG. 1. Amplification of Tc^r and Cm^r in DW1021 (*recA*$^+$) and DW1030 (recA). Cells were grown in L broth supplemented with chloramphenicol (A and C) or tetracycline (B and D) at 5 μg/ml for 200 generations at 37°C and then in drug-free medium (arrows) for another 100 generations. Symbols denote levels to which >80% of survivors grew on chloramphenicol-supplemented or tetracycline-supplemented L agar.

Pc^r and Ap^r, Tet mutants expressed more Kf^r than did Cml mutants, and all mutants were generally two to threefold more resistant to cephalothin than to ampicillin.

Pu^r was amplified to very high levels in all mutants, and the levels of resistance attained were independent of tetracycline or chloramphenicol selection, but somewhat dependent on *recA* function to achieve the higher levels of resistance. Rf^r essentially did not amplify during the transition from low to high Tc^r or Cm^r. It remained at the level reached in the low-level Tet and Cml mutants and was lower in *recA* than in *recA*$^+$ strains. The parent strains were already nalidixic acid-resistant but the resistance level was augmented by 10 to 80% in the amplified mutants. The possibility that existing nalidixic acid resistance might augment or promote the amplification of multiple resistance phenotypes was examined. The nalidixic acid-sensitive strain GMS407 was amplified with tetracycline or chloramphenicol and the resultant mutants were screened for other resistance phenotypes by the gradient plate method. Pc^r, Pu^r, and Rf^r phenotypes were amplified to levels comparable to those of the Na^r DW1021 and DW1030 strains (data not shown). Na^r reached levels of at least 40 μg/ml.

Since plasmid-mediated Tc^r is associated with low resistance to minocycline (MIC of minocycline/MIC of tetracycline < 0.1) (28), we tested these strains for Mc^r. We found that the ratio of the MIC of minocycline to the MIC of tetracycline was never less than 0.4; in fact, when chloramphenicol was the selective agent, the ratio approached 1.0. These results indicated a clear distinction between this resistance phenotype and that seen in plasmid-mediated Tc^r (28).

It has been reported that autoclaved chlortetracycline is a gratuitous inducer of plasmid-mediated Tc^r (5). With autoclaved chlortetracycline, we could not achieve induction or amplification of Tc^r in sensitive strains, nor could we maintain otherwise amplified Tc^r or Cm^r in resistant strains. However, when DW1021 and DW1030 were grown for 200 generations in the presence of a subinhibitory level of chlortetracycline (5 μg/ml after selection of first-step mutants), the resistance levels reached in DW1021 and DW1030 mutants were, respectively, 68 and 66 μM chlortetracycline, 63 and 74 μM minocycline, 55 and 51 μM tetracycline, and 257 and 245 μM chloramphenicol. These values were not very dissimilar from those in the high-level Tet or Cml mutants in Table 3. However, one striking difference was the high ratio of Mc^r/Tc^r (1.3) in the chlortetracycline-selected mutants compared with a Mc^r/Tc^r ratio of 0 · 5 in tetracycline-selected mutants. Indeed, chlortetracycline or chloramphenicol selection elaborated a higher Mc^r/Tc^r ratio than did tetracycline selection.

Growth rates. Doubling times of low-level Tet and Cml mutants were the same as that of the wild-type parent in drug-free L broth at 37°C. However, most intermediate- and high-level mutants grew noticeably slower under nonselective conditions, with doubling times up to twice that

of the parent strain. When Tet and Cml mutants were grown in L broth supplemented with antibiotics at 10% of the MICs, there was no effect on doubling times, but at 25% of the MICs, the mean generation time was twice as long. High-level Tc^r and Cm^r were maintained by growing cells in sublimiting drug concentrations (≥10% of the MIC).

Temperature sensitivity of resistance phenotypes and mucoidy. Stepwise amplification of resistance to tetracycline and chloramphenicol on agar plates supplemented with antibiotic was not observed at 42°C in three strains so tested (DO-23, DW1021, and DW1030). Low-level Cml mutants (5 to 10 μg/ml) were selected after several days at 42°C, but resistance to chloramphenicol at 20 μg/ml was unattainable. A slight smear covered plates supplemented with tetracycline at 5 μg/ml, but discrete colonies were not isolated. Furthermore, established high-level Tet and Cml mutants (previously selected at 37°C) did not grow at 42°C on the same or lower antibiotic concentrations, but when these plates were transferred to 37°C, colonial growth appeared within 24 h.

Although there was no loss of viability of mutants in the range 30 to 42°C, the magnitude of Tc^r or Cm^r was 40 to 60% higher at 30°C than at 37°C. This recognition of a temperature sensitivity of resistance phenotypes led us to consider the effect of temperature on the selection of mutants. The rate of spontaneous mutation to $Tc5^r$ or $Cm5^r$ was about 1.5 times higher at 30°C than at 37°C, but, as at 37°C, the lower temperature did not produce single-step mutants that expressed $Tc10^r$.

Occasional low- and high-level Tet or Cml mutants formed mucoid colonies on rich media at 37°C. This mucoidy was not elaborated at 42°C on drug-free rich or minimal plates, although the strains grew well under these conditions. Tc^r or Cm^r of mucoid cells was also temperature sensitive on drug-supplemented media.

Fusaric acid resistance. Fusaric and quinaldic acids are lipophilic chelating compounds that have been shown to be inhibitory to plasmid- or transposon-mediated tetracycline-resistant cells (5). They have been used as selective agents for tetracycline-sensitive clones from predominantly tetracycline-resistant populations (5, 23). When 10^6 cells of the Tn*10*-containing strains K0635 (*tna-7*::Tn*10*) (from A. Wright) and D1-188 (Tn*10* on R222 in D0-23) were plated on quinaldic acid minimal plates, 1.3×10^{-5} and 1.6×10^{-5} of colonies were recovered for K0635 and D1-188, respectively, and >90% of these were tetracycline sensitive. We applied this method to three D0-23 Tet mutants, AG101 ($Tc5^r$), AG103 ($Tc20^r$), and AG111 ($Tc100^r$). When we tested the effect of amplifiable Tc^r on quinaldic acid sensitivity we found that sensitivity increased with increasing Tc^r, thus, 78% of AG101, 37% of AG103, and 8% of AG111 colonies survived on quinaldic acid selective plates, but these colonies were still tetracycline resistant. Comparable results were achieved on fusaric acid-supplemented plates. The fusaric acid sensitivity of these mutants was clearly dissimilar from plasmid-mediated Tc^r.

Genetic basis of resistance. Representative strains (D0-21, D0-22, D0-23, DW1030, PA309, and AR101) were screened for plasmids after appropriate selection and amplification, by two methods (18, 32), but plasmid DNA bands were not detected on 0.7% agarose gels; nor could we observe resistance transfer in conjugal filter matings (13). Also, assays for chloramphenicol acetyltransferase and β-lactamase failed to detect enzymatic activity characteristic of most plasmid-determined Cm^r or Pc^r.

Non-transducibility of resistances. With the exception of the *cmlA* mutation, we were unable to obtain low- or high-level Tet or Cml transductants even at a frequency of 10^{-11}/PFU with P1 lysates prepared from Tet and Cml mutants. Auxotrophic markers were routinely transduced by these P1 lysates at frequencies of 10^{-6} to 10^{-4}/PFU. The non-transducibility of resistances might suggest that a cooperativity between dispersed loci was involved, or that the resistance phenotypes were refractory to direct selection.

Antibiotic transport. Accumulation of antibiotics by sensitive and resistant cells was examined in the presence or absence of the uncouplers DNP and carbonyl cyanide-*m*-chlorophenyl hydrazone, which are known to collapse the proton motive force across bacterial membranes (35). Sensitive cells actively accumulated tetracycline, whereas the resistant mutants AG102 and AG122 showed a markedly lower accumulation (Fig. 2). The reduced accumulation of tetracycline by the low-level Tet mutant AG102 disappeared above 600 μM tetracycline, whereupon an active (DNP-sensitive) uptake of the drug occurred (data not shown). In the intermediate-level Tet mutant AG106, however, an active uptake of tetracycline did not occur even at 800 μM tetracycline. When 2 mM DNP (Fig. 2) or 25 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (data not shown) was added, or when the endogenous energy supply was depleted by starvation (data not shown), the accumulation of tetracycline in the resistant cells increased to the level found in sensitive cells under these same energy-depleted conditions. The lipophilic tetracycline analog minocycline also exhibited an energy-dependent (DNP- or carbonyl cyanide-*m*-chlorophenyl hydrazone-

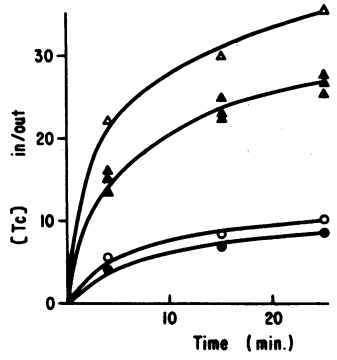

FIG. 2. Accumulation of 5 μM [^{3}H]-tetracycline by cells in assay buffer at 35°C. Symbols: AG100 (△), AG102 (○), AG122 (●), and the three strains preincubated with 2 mM DNP (▲).

inhibited) reduced accumulation in Tet and Cml mutants.

Inner membrane everted vesicles of the Tet mutants AG102 and AG106 showed an energy-dependent uptake of tetracycline at an external concentration of 10 μM (Fig. 3C and D). Moreover, the apparent strength of this uptake was twofold greater in the more resistant AG106 mutant. These findings demonstrated that an efflux system, previously cryptic, was chromosome mediated. Under these experimental conditions it compared quite favorably with plasmid R222-mediated tetracycline uptake (Fig. 3B). In contrast, sensitive AG100 vesicles accumulated an amount of tetracycline consistent with equilibration of internal and external levels and were unaffected by the presence of exogenous energy (Fig. 3A). That vesicles from sensitive cells did not actively accumulate tetracycline was an indication that the preparation largely contained everted vesicles, since right-sided vesicles, like intact cells, are known to accumulate tetracycline actively (25, 26).

To determine whether tetracycline and chloramphenicol were sharing the same efflux system we performed competition experiments. Increasing chloramphenicol to 600 μM had no effect on the reduced accumulation of 5 μM tetracycline in resistant cells (data not shown).

Since tetracycline resistance was temperature sensitive, we examined the effect of temperature on the energy-dependent efflux of this antibiotic. When AG102 cells that had been grown at 35°C and initially assayed for tetracycline incorporation at 35°C were shifted to 42°C, a rapid increase in accumulation of tetracycline occurred; this accumulation approached that seen in AG100 sensitive cells (Fig. 4). Significantly, tetracycline accumulation in sensitive cells with or without DNP did not deviate after the shift to 42°C (Fig. 4). Thus, lower resistance to tetracy-

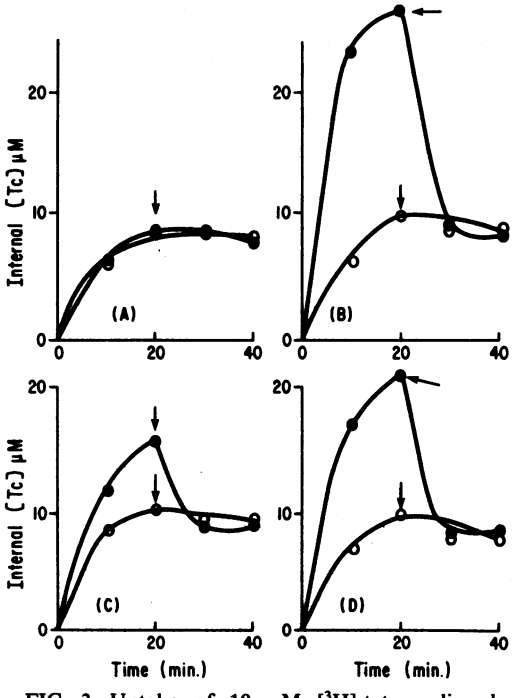

FIG. 3. Uptake of 10 μM [^{3}H]-tetracycline by everted membrane vesicles in assay buffer at 30°C. Panels: (A) AG100 vesicles, (B) D1-188 (AG100 with plasmid R222) vesicles, (C) AG102 vesicles (low-level Tc^r), and (D) AG106 vesicles (high-level Tc^r). Vesicles were allowed to accumulate tetracycline for 20 min in the presence (●) or absence (○) of 20 mM D-lactate; 2 mM DNP was added (arrows), and incubation was continued.

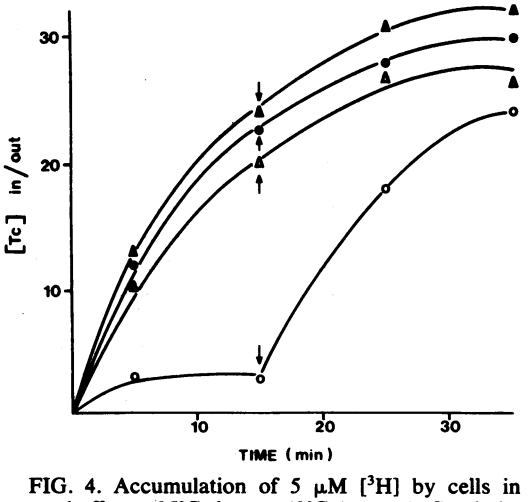

FIG. 4. Accumulation of 5 μM [^{3}H] by cells in assay buffer at 35°C then at 42°C (arrows). Symbols: AG100 without (▲) and preincubated (△) with 2 mM DNP; AG102 without (○) and preincubated (●) with DNP.

cline at 42°C correlated with higher uptake at 42°C.

We also performed [1-^{14}C]penicillin transport assays. Unlike the reduced accumulation of tetracycline seen in the Tet and Cml mutants, there was no detectable reduction in uptake of penicillin G by resistant cells. In the presence of 5 to 25 μM external penicillin G, sensitive and resistant cells showed the same accumulation profiles, and these were unaffected by the addition of DNP (data not shown). Similar studies were performed with [^{14}C]chloramphenicol. Chloramphenicol probably enters the cell by passive diffusion (16; unpublished results). Although resistant cells showed an energy-dependent and DNP-sensitive reduced accumulation of the drug, assays with everted membrane vesicles did not show an increase in uptake of [^{14}C]chloramphenicol in the presence of exogenous energy.

DISCUSSION

We have presented evidence that Tet and Cml phenotypes from a number of plasmidless *E. coli* K-12 and wild-type strains were amplifiable to high levels. The resistance and amplification of resistance were temperature sensitive. Although tetracycline or chloramphenicol selection produced collateral resistance to many unrelated antibiotics, Tc^r was mediated partially or wholly by a newly detected efflux system for this antibiotic. The bases of collateral resistance to the other antibiotics have not yet been determined.

Chromosomal resistances are often expressed from single genes, but occasional cooperativity between loci has been demonstrated (36, 38). Azaserine resistance in *E. coli* involves mutations at three loci that arise spontaneously and sequentially and promote independent or additive resistance (38). Low-level resistance to tetracycline, chloramphenicol, and penicillin G in *Neisseria gonorrhoeae* has been linked to interactive chromosomal loci (36).

In the accompanying paper (14), we demonstrate that four regions of the *E. coli* K-12 chromosome contribute to the resistance phenotypes, and that a single pleiotropic locus at 34 min is required for the expression of all of the resistances described in these mutants.

The association of chromosomally mediated Tc^r and Cm^r is not unique to gram-negative species. In a review of treatment failure (37), Cm^r and Tc^r *Staphylococcus epidermidis* organisms were isolated after sequential therapeutic administration of methicillin, gentamicin, and chloramphenicol. Although some isolates were sensitive to chloramphenicol and tetracycline, testing in vitro resulted in the sensitive isolates becoming resistant to the same levels of chloramphenicol and tetracycline as the resistant isolates at frequencies of 10^{-6} and 5×10^{-9}, respectively. Resistance was stable, and Tc^r and Cm^r were coincident without alteration of the sensitivity of the organism to a wide range of other antibiotics. Our observations with *E. coli* K-12 concur with this cited study in establishing a relatively high spontaneous rate of mutation to Cm^r and a low rate of mutation to Tc^r (Table 2).

Serial transfer of field isolates of *Salmonella typhimurium* and *E. coli* in chlortetracycline produced Mc^r/Tc^r ratios of nearly 2 (17). We also found a Mc^r/Tc^r ratio higher than unit upon amplification with chlortetracycline. Moreover, in all Tet and Cml mutants the Mc^r/Tc^r ratio was higher than in cells harboring plasmid-borne tetracycline resistance determinants (28).

Spontaneous mutants of *E. coli* K-12 resistant to a high level of ampicillin have been obtained by serial transfer of cells in increasing concentrations of ampicillin (10). These mutants hyperproduced β-lactamase through multiple repetition of the chromosomal *ampA-ampC* region. In contrast, although tetracycline or chloramphenicol selection produced high-level resistance to penicillin G, ampicillin, and cephalothin (Table 3), we did not detect β-lactamase activity. Since there was no detectable change in penicillin G transport, another mechanism must be involved, such as changes in the penicillin-binding proteins of the cell envelope.

Growth in subinhibitory drug concentration favors mutation and selection in allowing the original, susceptible strain to continue to grow at some minimal level (20). Similarly, the opportunity for cells carrying favorable mutations to outgrow other types is conditional on continued growth under selective conditions. Autoclaved chlortetracycline did not select or maintain resistance, a finding that suggested that the structural integrity or antibacterial activity of the drug molecule was an absolute requirement for amplification. Although continuous tetracycline or chloramphenicol selection produced high-level resistance to both agents (Fig. 1) and to many other classes of antibiotics, there was no change in aminoglycoside sensitivity (Table 3). Repeated transfer of *E. coli* and *Staphylococcus aureus* in subinhibitory concentrations of gentamicin produced resistance to all aminoglycosides, but not to ampicillin, chloramphenicol, or tetracycline (15). This resistance was ascribed to an aminoglycoside-specific modification in permeability or transport. If Tet and Cml mutants had incorporated nonspecific permeability barriers, such as changes in outer membrane porins, one would expect that aminoglycoside resistance would also be enhanced (12).

Transport assays with everted inner membrane vesicles provided evidence for the presence of an energy-driven tetracycline efflux sys-

tem in Tet and Cml mutants (Fig. 2, 3, and 4). The efflux was related to the tetracycline resistance phenotype in that both were temperature sensitive. This mechanism for resistance resembles that mediated by plasmids, except that it mediates higher levels of minocycline resistance and is not induced or maintained by autoclaved chlortetracycline.

Since amplification to high-level Tc^r and Cm^r was *polA* and *recA* independent, duplication of mutant alleles by illegitimate recombination (1) mediated by insertion sequences or rearrangements is a possible mechanism of amplification. In the absence of selective pressure, high-level Tc^r and Cm^r was lost (Fig. 1), but even after 300 generations low-level Tc^r and Cm^r components remained. Growth in the absence of drug challenge is likely to favor the loss of these mutations, but if the resistance mechanism produces little burden on its host, the reversal of all mutations may require very long times (20).

These findings provoke speculation about the origins of extrachromosomal resistance determinants. It is not unlikely that these were derived from primal chromosomal genes by the differentiation of gene functions needed to meet the challenge of hostile environments, particularly in the niche of antibiotic-producing soil microbes (20). It is plausible that resistance mechanisms arose through a series of mutations. This slow evolution of host resistance mechanisms might then have been followed by the mobilization of resistance alleles (transposons, plasmids), and the original chromosomal mutations were not completely lost, but became vestigial or latent.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI16756 from the National Institutes of Health and by grant MV73E from the American Cancer Society.

We thank L. McMurry for help with the vesicle studies.

LITERATURE CITED

1. **Anderson, R. P., and J. R. Roth.** 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. **31**:473–505.
2. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K-12. Genetics **39**:440.
3. **Azemun, P., T. Stull, M. Roberts, and A. L. Smith.** 1981. Rapid detection of chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **20**:168–170.
4. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
5. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
6. **Braude, A. I., J. Banister, and N. Wright.** 1954–1955. Use of the gradient plate for routine clinical determinations of bacterial sensitivities to antibiotics. Antibiotics Annual, p. 1133–1140.
7. **Butlin, J. D., G. B. Co, and F. Gibson.** 1971. Oxidative phosphorylation in *Escherichia coli* K-12. Biochem. J. **124**:75–81.
8. **Cavalli, L. L.** 1952. Genetic analysis of drug resistance. Bull. W.H.O. **6**:185–206.
9. **Cavalli, L. L., and G. A. Maccacaro.** 1952. Polygenic inheritance of drug-resistance in the bacterium *Escherichia coli*. Heredity **6**:311–331.
10. **Cole, S. T., and J. R. Guest.** 1979. Production of a soluble form of fumarate reductase by multiple gene duplication in *Escherichia coli* K-12. Eur. J. Biochem. **102**:65–71.
11. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
12. **Davies, J., and D. I. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–518.
13. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn916) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.
14. **George, A. M., and S. B. Levy.** 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J. Bacteriol. **155**:541–548.
15. **Gialdroni Grassi, G.** 1979. Drug-inactivating enzymes of bacteria grown in subminimal inhibitory concentrations of antibiotics. Rev. Infect. Dis. **1**:852–857.
16. **Hurwitz, C., and C. B. Braun.** 1967. Measurement of binding of chloramphenicol by intact cells. J. Bacteriol. **93**:1671–1676.
17. **Jarolmen, H., D. Hewel, and E. Kain.** 1970. Activity of minocycline against R factor-carrying *Enterobacteriaceae*. Infect. Immun. **1**:321–326.
18. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
19. **Kilbane, J. J., and M. H. Malamy.** 1980. F factor mobilization of nonconjugative chimeric plasmids in *Escherichia coli*: general mechanisms and a role for site-specific *recA*-independent recombination at *oriV1*. J. Mol. Biol. **143**:73–93.
20. **Koch, A. L.** 1981. Evolution of antibiotic resistance gene function. Microbiol. Rev. **45**:355–378.
21. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
22. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
23. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**:1110–1112.
24. **McMurry, L. M., J. C. Cullinane, and S. B. Levy.** 1982. Transport of the lipophilic analog minocycline differs from that of tetracycline in susceptible and resistant *Escherichia coli*. Antimicrob. Agents Chemother. **22**:791–799.
25. **McMurry, L. M., J. C. Cullinane, R. E. Petrucci, Jr., and S. B. Levy.** 1981. Active uptake of tetracycline by membrane vesicles from susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **20**:307–313.
26. **McMurry, L. M., and S. B. Levy.** 1978. Two transport systems for tetracycline in sensitive *Escherichia coli*: critical role for an initial rapid uptake system insensitive to energy inhibitors. Antimicrob. Agents Chemother. **14**:201–209.
27. **McMurry, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:3974–3977.
28. **Mendez, B., C. Tachibana, and S. B. Levy.** 1980. Heterogeneity of tetracycline resistance determinants. Plasmid **3**:99–108.

29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Novel, M., and G. Novel.** 1976. Regulation of β-glucuronidase synthesis in *Escherichia coli* K-12: constitutive mutants specifically derepressed for *uidA* expression. J. Bacteriol. **127**:406–417.
31. **Park, C. H., J. S. Lopez, and C. B. Cook.** 1978. Acidometric agar plate method for ampicillin susceptibility testing of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **13**:318–320.
32. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
33. **Reeve, E. C. R.** 1968. Genetic analysis of some mutations causing resistance to tetracycline in *Escherichia coli* K-12. Genet. Res. **11**:303–309.
34. **Reeve, E. C. R., and D. R. Suttie.** 1968. Chromosomal location of a mutation causing chloramphenicol resistance in *Escherichia coli* K-12. Genet. Res. **11**:97–104.
35. **Rosen, B. P., and E. R. Kashket.** 1978. Energetics of active transport, p. 567. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
36. **Sparling, P. F., F. A. Sarubbi, Jr., and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124**:740–749.
37. **Sugarman, B., and E. Pesanti.** 1980. Treatment failures secondary to *in vivo* development of drug resistance by microorganisms. Rev. Infect. Dis. **2**:153–168.
38. **Williams, M. V., T. J. Kerr, R. D. Lemmon, and G. J. Tritz.** 1980. Azaserine resistance in *Escherichia coli*: chromosomal location of multiple genes. J. Bacteriol. **143**:383–388.
39. **Winkler, H. H., and T. H. Wilson.** 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200–2211.